TISSUE PROTECTIVE CYTOKINES FOR THE TREATMENT AND PREVENTION OF SEPSIS AND THE FORMATION OF ADHESIONS

FIELD OF THE INVENTION

The present invention is directed to a method of treating, preventing, delaying the onset, and/or reducing the effects of sepsis and related complications. In particular, the present invention is directed to the use of tissue protective cytokines for the treatment, prevention, delay, and/or reduction of complications with regard to sepsis, adhesion formation, and organ failure. Furthermore, the tissue protective cytokines of the present invention are also contemplated for treatment, prevention, delay, and/or reduction of complications of general inflammatory conditions resulting from infection.

BACKGROUND OF THE INVENTION

Several strategies exist for responding to infection, immune challenges, inflammation, and trauma in a host. One mechanism by which the host attempts to respond to these challenges is through the upregulation of cytokines, nonantibody proteins that act as intercellular regulators. Some cytokines, known as proinflammatory cytokines, counteract the challenges to the host by enhancing the disease in the hopes of ridding the host of the challenge and host cells damaged by the challenge. Proinflammatory cytokines include, but are not limited to, interleukins (IL), such as IL-1, IL-6, IL-8, and IL-18, and tumor necrosis factor (TNF).

When released, the proinflammatory cytokines have the effect at the site of injury of increasing the release of antibodies and their compliments, T and B cell activation, the adhesion of platelets to blood vessel walls, and extravascuarization of lymphocytes and macrophages. These changes lead to a localized environment at the site of injury including fever, tissue injury, tumor necrosis, induction of other cytokines and immunoregulation and apoptosis. This localized response is toxic not only to the source of the challenge to the host but also to the host cells within the penumbra of the proinflammatory cytokine response. Thus, it is not surprising that on a systemic level, such as may occur during overwhelming infection or serious trauma to the host, many of these proinflammatory cytokines are harmful to the host producing fever, inflammation, tissue destruction, and, in some cases, shock and death.

Representative of the action of the various proinflammatory cytokines is TNF. TNF is a proinflammatory cytokine produced by many cell types, including macrophages, monocytes, lymphoid cells and fibroblasts in response to inflammation, infection, and other environmental challenges. TNF elicits a wide spectrum of cellular responses, including fever, shock, tissue injury, tumor necrosis, anorexia, induction of other cytokines and immunoregulatory molecules, cell proliferation, differentiation and apoptosis. When released TNF has an effect at the site of injury of increasing the release of antibodies and their compliments, T and B cell activation, the adhesion of platelets to blood vessel walls, and extravascuarization of lymphocytes and macrophages. Systemically, TNF acts upon the hypothalamus and liver. TNF stimulates the hypothalamus to release corticotropin releasing hormone, suppress appetite and induce fever. In response to TNF, the liver initiates an acute phase response resulting in the synthesis of several proteins including C-reactive protein, coagulation factors and compliment factors. Also, TNF induces insulin resistance. In the defined area of injury or infection, TNF is vital to removing the particular infectious agent and adapting the body's immune response to the particular injury.

On a systemic level, however, in which TNF as well as other proinflammatory cytokines may be present at higher concentrations or for prolonged times, TNF can have deleterious effects on the body. At high concentrations TNF activates an IL-1 & Il-6 cascade that results in cachexia (wasting). Additionally, TNF can lead to systemic edema, hypoproteinemia, and neutropenia which can result in disseminated extravascular coagulation and eventually multiple organ failure. In chronic diseases such as cancer, TNF can also interfere with vital endogenous functions within the host. For example, TNF may interfere with the ability of endogenous erythropoietin to maintain the hematocrit of the host, leading to a condition referred to as the anemia of chronic diseases (ACD). A typical course of treatment with recombinant erythropoietin may not counteract the effects of the proinflammatory cytokine, thereby requiring the administration of elevated doses of recombinant erythropoietin just to maintain the normal hematocrit of the host. Beyond the additional costs associated with the increased dosing, there is also the risk of adverse side effects from the increased doses of erythropoietin such as thrombosis. In addition to the conditions detailed below, proinflammatory cytokines, including, but not limited to, TNF are associated with diseases such as chronic inflammation, bacterial septic shock, bacterial toxic shock, graft vs. host disease, and HIV infection and AIDS.

Sepsis

Sepsis is the body's response to any kind of infection, *e.g.*, bacterial, viral, parasitic, or fungal. Sites of infection are typically the lungs, the urinary tract, the abdomen, and the pelvis. In some cases, however, the actual site of infection cannot be detected. Although sepsis was once thought to be a systemic inflammatory response, it is now recognized that sepsis also includes prothrombotic diathesis and impaired fibrinolysis.

Once sepsis commences, widespread inflammation and clotting occurs throughout the body. Whereas in a healthy body, immune modulators would be released to fight the infection and heal the body, in sepsis, an overabundance of immune regulators is released. The release of proinflammatory cytokines such as TNF, interleukin-1, and interleukin-18 lead to the inflammation of endothelial linings, elevation of the core temperature, loss of appetite, and anemia. In addition, inflammation of the lining of blood vessels activates the blood clotting process. Because sepsis decreases the body's natural production of protein C, which regulates blood clotting and controls inflammation, the body's ability to break down the formed blood clots is suppressed. This suppression leads to clotting in vital organs, limbs, fingers, and toes, which, in turn, leads to organ failure or gangrene.

Sepsis may present itself in varying degrees. For example, in cases of severe sepsis, which occurs when acute organ dysfunction or failure results, the body's normal defense reaction goes into overdrive, setting off a cascade of events that can lead to widespread inflammation and blood clotting in tiny vessels throughout the body. Septic shock occurs when a patient with severe sepsis experiences cardiovascular system failure. This failure causes the blood pressure to drop, which, in turn, deprives vital organs of an adequate oxygenated blood supply. Septicemia is a sepsis that has an infection in the bloodstream itself. In fact, septicemia may cause ischemia, *i.e.*, poor blood supply to at least one organ. For example, when blood flow to the kidneys is reduced to dangerously low levels for substantial time period, ischemic acute renal failure (ARF) may develop. The depressed blood flow also results in necrosis, or tissue death, in affected organs.

Providing the source of the sepsis can be identified, many cases of sepsis will respond to treatment. Once isolated, a treatment regime specific to the cause of infection is initiated. Known treatment includes the use of antibiotics, surgical excision of infected or necrotic tissues, drugs that increase activated protein, and steroids (in cases of septic shock). For example, a typical course of sepsis treatment includes administration of a broad spectrum antibiotic until the cause of infection is isolated. However, the mortality rate of sepsis

patients remains relatively high in cases of sepsis where the cause and/or area of infection is not ascertainable.

Depending on the severity of sepsis, anti-infection agents, draining techniques, fluids, drugs to raise the mean arterial blood pressure (MAP) such as norepinephrine and phenylephrine, drugs to improve renal function such as dopamine, drugs to increase oxygen delivery and oxygen consumption such as dobutamine and epinephrine, mechanical ventilators to support breathing, and dialysis for kidney failure may be used in the course of treatment. In addition, pharmacological agents that have been shown to have beneficial effects on immune responses following shock and sepsis include ATP-$MgCl_2$, nonanticoagulant heparin, calcium channel blockers, chloroquine, cyclooxygenase inhibitors, PAF antagonists, anti-inflammatory cytokines, growth factors, dietary manipulation, anti-TNF antibodies, activated protein C (Xigris®, Eli Lilly, Indianapolis, Indiana), and sex hormones. Recovery from sepsis is greatest when the condition is quickly diagnosed and promptly treated.

Recombinant erythropoietin (rhu-EPO), commercially available under tradenames PROCRIT® (from Ortho Biotech Inc., Raritan, NJ), EPOGEN® (from Amgen, Inc., Thousand Oaks, CA), and NEORECORMON (from Roche, Basel, Switzerland)has also recently been investigated with regard to treatment of various conditions related to sepsis. In addition, U.S. Patent Publication No. 2003/0083251 generally discloses the use of rhu-EPO to aid in the regeneration of renal tubular cells and prevention of apoptosis of the renal tubular cells in order to treat patients with ischemic ARF. Furthermore, US Patent Publication No. 2002/0061849 generally discloses the use of rhu-EPO to aid in the treatment of inflammation in a non-ischemic condition in one or more organs. However, because of erythropoietin's erythropoietic effects -- increased hematocrit, vasoconstriction, hyperactivation of platelets, pro-coagulant activity, and increased production of thrombocytes -- treatment with rhu-EPO poses additional risks given the widespread clotting in vital organs, limbs, fingers, and toes that is associated with sepsis.

Adhesions.

In addition to sepsis, proinflammatory cytokines, such as TNF, have been associated with the formation of adhesions, abnormal fibrous bands or connections between organs and other structures of the body, as well. Adhesions may be a complication of, or related to,

sepsis but also may occur independently. For example, adhesions may form as a result of surgery, trauma, infection, chemotherapy, and radiation. In fact, adhesions are almost an inevitable outcome of surgery, *i.e.*, about 93 percent of patients who have undergone abdominal surgery suffer from adhesions to some degree (compared with adhesion formation in about 10.4 percent of patients who had never undergone a previous abdominal operation). *See* D. Menzies and H. Ellis, *Intestinal Obstruction from Adhesions—How Big is the Problem?*, ANN. R. COLL. SURG. ENGL. 72: 60-3 (1990).

The formation of adhesions can cause severe pain and apply unnatural pressure or tension on organs or other structures of a patient. For example, adhesions in the abdominal region of the body may cause the intestines of a patient to become trapped or squeezed between organs or other structures of the body. In some cases, the intestines may become blocked or significantly obstructed due to nearby adhesions.

The formation of these abnormal connections between two parts of a body leads to a host of other conditions. For example, as cesarean sections are becoming a more common method of childbirth, women who undergo this major abdominal surgery are likely to form adhesions and, as a result, experience chronic pelvic pain. In addition, adhesions involving female reproductive organs may lead to infertility and dyspareunia.

A number of agents have been researched in connection with preventing and treating adhesions, *e.g.*, dextran, corticosteroids, phosphatidylcholine, phospholipase inhibitors, non-steroidal anti-inflammatory drugs, proteoglycans, heparin, and tissue plasminogen activator. *See, e.g.*, C.L. Kowalczyk and M.P. Diamond, *The Management of Adhesive Disease, in* PERITONEAL ADHESIONS 315-324 (K.H. Treutner and V. Schumpelick, eds., 1997). Some, but not all of these agents, are believed to be effective in the treatment of adhesions because of their ability to interfere with coagulation and fibrinolysis. Clinical experience with the majority of these agents, however, is limited due to bleeding complications. In addition, hyaluronic acid derivatives have been shown to prevent postsurgical adhesions, particularly in the intra-abdominal area. *See, e.g.*, J.M. Becker *et al., Prevention of Postoperative Abdominal Adhesions by a Sodium Hyaluronate-based Bioresorbable Membrane: A Prospective, Randomized, Double-blind Multicenter Study, in* J. AM. COLL. SURG. **183** 297-306 (1996). Furthermore, beta-glucan, which is a glucose polymer that binds with high affinity to the receptors on monocytes and neutrophils in a competitive manner, has been shown to have a reducing effect on the frequency of adhesion after experimentally developed intraabdominal sepsis in Wistar rats. A. Bedirli *et al., Prevention of Intraperitoneal Adhesion*

Formation Using Beta-Glucan After Ileocolic Anastomosis in a Rat Bacterial Peritonitis Model, in AM. J. SURG. **185** 339-343 (2003).

Surgery may also be used as a course of treatment for adhesions. Generally, a physician will perform surgery to sever the adhesions from the organ or other part of the body. Given that adhesions are often a complication of surgery, however, surgery to remove adhesions frequently results in the formation of new adhesions. While some surgical procedures involve placement of sleeves over organs adjacent to the areas affected by the surgery and thus, help to prevent adhesions involving these organs, such procedures have had mixed results. In addition, the organ sleeves also require additional surgery to remove the sleeves.

Thus, despite the increased awareness with regard to adhesions, research into treatment methods have met with limited success. Many physicians are unwilling or unable to address the treatment of adhesions and many insurance companies are unwilling to pay for treatments that are, at best, marginally successful.

Wound Healing.

Healing is an essential process of the body that reestablishes the integrity of damaged tissue. This process is often viewed in terms of wounds, ulcers or lesions of the skin resulting from various causes such as trauma, surgery, pressure (bed sores), burns, diabetes, etc. The severity of the wounds is characterized by the extent the wound penetrates the skin. Stage I wounds are characterized by redness or discoloration, warmth, and swelling or hardness. Stage II wounds, partial thickness wounds, penetrate the epidermis and superficial dermis of the skin. Stage III wounds, full thickness wounds, penetrate through the dermis of the skin but do not penetrate the membrane separating the skin from deeper organs. Stage IV wounds involve damage to the underlying muscle or bone.

Although all wounds heal through the same process: inflammation, epithelialization, angiogenesis, and the accumulation of matrix; the ease with which the wound heals is largely based on the severity of the wound and the health of the wounded individual. In general, Stage I and Stage II wounds heal through the regeneration of epithelial cells by the underlying dermis. Whereas, Stage III and IV wounds heal through the production of a scar. Proinflammatory cytokines, such as TNF, play a role in the healing of wounds, however, it is speculated that TNF may have an adverse effect on the accumulation of collagen in the

healing wound and ultimately on the time the wound takes to heal and the strength of the repaired tissue.

Several therapeutics as well as therapeutic methods have been developed to assist the body in healing wounds. Several compounds are considered to have a therapeutic effect on wound healing including, but not limited to, growth factors (epidermal growth factor, Insulin-like Growth Factor, human growth hormone, fibroblast growth factor, vascular endothelial growth factor, interleukin-6, and interleukin-10), nutritional supplements (arginine, glutamine, vitamin C, vitamin B5, Bromelain, Curcumin, zinc, copper), and herbal supplements (aloe vera, Centella). Furthermore, various therapeutic methods including, but not limited to, hyperbaric oxygen therapy, whirlpool therapy, ultrasound therapy, electrical stimulation, and magnetic therapy have been utilized to aid the body in healing wounds.

If a wound does not heal properly or fails to heal at all it can lead to several complications chief among them scarring and infection. Depending upon the severity of the wound, the body may generate scar tissue in healing the wound. Aside from the aesthetic concerns of a scar, the scar may impair movement of the individual depending upon its severity. Additionally, a wound presents an opportunity for bacteria and other infectious agents to enter the body. Depending upon the severity of infection it may spread and become systemic leading to sepsis or septicemia.

Rhu-EPO has also been investigated for its possible healing effects in rat models of random ischemic flaps. For example, rhu-EPO has been shown to reduce necrosis, decrease neutrophil infiltration, and prevent increased temperature with regard to ischemic skin flap injuries. *See* M. Buemi *et al.*, *Recombinant Human Erythropoitein Influences Revsacularization and Healing in a Rat Model of Random Ischaemic Flaps*, ACTA DERM VENEREOL, 82: 411-417 (2002). This finding suggests that rhu-EPO administration can improve the wound healing process, both in early and late stages of injury, by reducing the inflammatory response, increasing the density of capillaries in ischemic flaps and allowing earlier repair of a damaged area. However, as mentioned above, because rhu-EPO has erthyropoietic activity, the use of rhu-EPO for treatment of these conditions may cause a greater degree of clotting or complications than already initiated by the healing process.

In sum, no one agent or treatment strategy has demonstrated sufficient value for the management of sepsis cases, the incidence of sepsis, the formation of adhesions, wound healing or general inflammatory conditions. In fact, the mortality associated with sepsis and related conditions remains high. Every year, approximately 215,000 people die from severe sepsis and one out of every three patients who develop severe sepsis will die within a month.

And, cases of sepsis are expected to rise in the future due to the increased awareness of the condition and sensitivity for the diagnosis, the number of immunocompromised patients, the use of invasive procedures, the number of resistant microorganisms, and the growth of the elderly population. In addition, the chronic pain associated with adhesions and general inflammatory conditions is often untreated due to the lack of a successful treatment strategy.

Thus, there exists a need in the art for method and therapeutics for treating, preventing, delaying the onset of, and reducing the effects of proinflammatory cytokines for the purposes of limiting the penumbra of their action and further addressing their systemic effect. In particular, a need exists for treating, preventing, delying the onset of, and reducing the effects of proinflammatory cytokines in conditions of sepsis, adhesions, wounds, chronic disease and general inflammatory conditions. In addition, it would be beneficial to provide methodoligies that have the ability to repair or prevent damage to tissue in ischemic conditions.

SUMMARY OF THE INVENTION

The present invention is directed to a method of treating, preventing, delaying the onset, and/or reducing the effects of sepsis, adhesions, general inflammatory conditions, and combinations thereof by administering at least one tissue protective cytokine in a therapeutically effective amount. In addition, the present invention relates to the prevention or reduction of scarring relating to injury and incisions using at least one tissue protective cytokine. The at least one tissue protective cytokine may be any tissue protective cytokine having tissue protective functionality. In one embodiment, however, the at least one tissue protective cytokine is a chemically modified EPO. In another embodiment, the chemically modified EPO is carbamylated EPO.

One embodiment of the present invention relates to a method of treating, preventing, delaying the onset of, or reducing the effects of proinflammatory cytokines in a mammal. Other embodiments relate to methods of treating, preventing, delaying the onset of a condition associated with an effect of proinflammatory cytokines. Some examples of conditions associated with the effects of proinflammatory cytokines include sepsis, adhesions, wounds, inflammation or chronic disease. These methods may involve the steps of administering a therapeutically effective amount of one or more tissue protective cytokines in a pharmaceutical carrier.

In addition, the present invention also is directed to pharmaceutical compositions that may be used in the methods described herein. For instance, one embodiment is directed

toward a pharmaceutical composition comprising an amount of at least one tissue protective cytokine effective in treating, preventing, delaying the onset of, or reducing the effects of proinflammatory cytokines in a mammal. Another embodiment is directed toward a pharmaceutical composition comprised of an amount of at least one tissue protective cytokine effective in treating, preventing, delaying the onset of a condition associated with proinflammatory cytokines in a mammal.

Some tissue protective cytokines used in the present invention may be chemically modified erythropoietin or mutated erythropoietin.

In some embodiments where a chemically modified erythropoietin is used, the chemically modified erythropoietin may include one or more of the following: i) an erythropoietin that lacks sialic acid moieties; ii) an erythropoietin having at least no sialic acid moieties; iii) an erythropoietin having at least no N-linked or no O-linked carbohydrates; iv) an erythropoietin having at least a reduced carbohydrate content by virtue of treatment of native erythropoietin with at least one glycosidase; v) an erythropoietin having at least one or more oxidized carbohydrates; vi) an erythropoietin having at least one or more oxidized carbohydrates and is chemically reduced; vii) an erythropoietin having at least one or more modified arginine residues; viii) an erythropoietin having at least one or more modified lysine residues or a modification of the N-terminal amino group of the erythropoietin molecule; ix) an erythropoietin having at least a modified tyrosine residue; x) an erythropoietin having at least a modified aspartic acid or a glutamic acid residue; xi) an erythropoietin having at least a modified tryptophan residue; xii) an erythropoietin having at least one amino group removed; xiii) an erythropoietin having at least an opening of at least one of the cystine linkages in the erythropoietin molecule; or xiv) a truncated erythropoietin. In another embodiment, the chemically modified erythropoietin lacks erythropoietin's erythropoietic effects. The chemically modified erythropoietin also may comprise carbamylated erythropoietin.

Similarly, in some embodiments involving a mutated erythropoietin, the mutated erythropoietin may be selected from one or more of the following mutations C7S, R10I, V11S, L12A, E13A, R14A, R14B, R14E, R14Q, Y15A, Y15F, Y15I, K20A, K20E, E21A, C29S, C29Y, C33S, C33Y, P42N, T44I, K45A, K45D, V46A, N47A, F48A, F48I, Y49A, Y49S, W51F, W51N, Q59N, E62T, L67S, L70A, D96R, K97D, S100R, S100E, S100A, S100T, G101A, G101I, L102A, R103A, S104A, S104I, L105A, T106A, T106I, T107A, T107L, L108K, L108A, S126A, F142I, R143A, S146A, N147K, N147A, F148Y, L149A, R150A, G151A, K152A, L153A, L155A, C160S, I6A, C7A, B13A, N24K, A30N, H32T,

N38K, N83K, P42A, D43A, K52A, K97A, K116A, T132A, I133A, T134A, K140A, P148A, R150B, G151A, K152W, K154A, G158A, C161A, or R162A. . In another embodiment, the mutated erythropoietin lacks erythropoietin's erythropoietic effects.

Two examples of proinflammatory cytokines are Interleukin and TNF. One or more effects of the proinflammatory cytokine may include fever, wasting, lethargy, anemia, edema, ischemia, organ failure and insulin resistance. Additional features and advantages of the present invention are described in greater detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features and advantages of the invention can be ascertained from the following detailed description that is provided in connection with the drawing(s) described below:

FIG. 1 is a graphical representation of the survival rate of Sprague Dawley rats after cecum ligation and puncture (CLP) and subsequent treatment with saline or a tissue protective cytokine of the invention;

FIG. 2 is a graphical representation of the adhesion score for Sprague Dawley rats following CLP and subsequent treatment with saline or a tissue protective cytokine of the invention;

FIG. 3 is a graphical representation of the illness score for Sprague Dawley rats subjected to CLP and subsequent treatment with saline or a tissue protective cytokine of the invention;

FIG. 4 is a graphical representation of the adhesion score for Sprague Dawley rats following CLP with and without sepsis introduction and subsequent treatment with saline and a tissue protective cytokine of the invention; and

FIG. 5 is a graphical representation of the serum TNF level for Sprague Dawley rats following CLP with and without sepsis introduction and subsequent treatment with saline and a tissue protective cytokine of the invention.

FIG. 6 is a chart demonstrating the core body temperature for Sprague Dawley rats treated with saline or a tissue protective cytokine after lippopolysaccharide (LPS) induced sepsis for a period of 24 hours.

FIGS 7 (a) and (b) are charts demonstrating the levels of IL-6 (Fig. 7a) or TNF (Fig 7b) in the serum of Sprague Dawley rats treated with saline or a tissue protective cytokine after LPS induced sepsis.

FIGS 8(a) and (b) are charts demonstrating the core body temperature of Sprague Dawley rats treated with a tissue protective cytokine peripherally (Fig. 8a) or centrally (Fig. 8b) after LPS induced sepsis.

FIG 9 is a graphic representation of the percentage of lesion healed in Sprague Dawley rats thirty-four (34) days after being subjected to an ischemic skin flap test and subsequently treated with saline or a tissue protective cytokine.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel compositions for the treatment, prevention, delay, or reduction of the effects of proinflammatory cytokines, such as TNF, in conditions including, but not limited to, sepsis and sepsis-related conditions, adhesions, wound healing, and chronic disease. The effects of the proinflammatory cytokines addressed by the tissue protective cytokines include, but are not limited to, fever, wasting, lethargy, anemia, edema, ischemia, organ failure and insulin resistance. The compositions of the invention are also contemplated for the treatment, prevention, delay, or reduction of the effects of inflammatory conditions in one or more organ(s) or tissue(s) resulting from infection, such as in the case of meningitis. In particular, the present invention is directed to compositions including tissue protective cytokines that are successful in the treatment of the effects of proinflammatory cytokines in conditions including sepsis, adhesions, wound healing, chronic disease, and inflammatory conditions.

In addition, the compositions of the inventions are useful in treating, preventing, and/or reducing the appearance of scarring from injury. For example, when a tissue protective cytokine of the present invention is used in conjunction with abdominal surgery, scarring may be substantially reduced. In one embodiment, the tissue protective cytokines of the present invention are used to prevent scarring from surgical incisions.

Compositions of the Invention

Any cytokine that exhibits tissue protective capability is contemplated for use with the present invention. The compositions of the invention may include erythropoietin. For example, a suitable tissue protective cytokine of the invention may be an EPO molecule, which may exist in a number of forms, *e.g.* α, β, asialo and others. The α and β forms have the same potency, biological activity, and molecular weight, but differ slightly in the

carbohydrate components, while the asialo form is an α or β form with the terminal sialic acids removed from the carbohydrate components.

Also, any tissue protective cytokine capable of treating, preventing, delaying the onset of, and/or reducing the effect of sepsis, sepsis-related conditions, and general inflammatory conditions is contemplated as well. As used herein, the term "tissue protective cytokines" refer to any cytokine that is a derivative of erythropoietin that possesses the tissue protective activity of erythropoietin. Preferably the tissue protective cytokine lacks at least one or more of erythropoietin's erythropoietic effects. Most preferably, the tissue protective cytokine lacks all of the erthropoietic effects of erythropoietin. For example, this may be accomplished by modifying erythropoietin through chemical or mutational processes that affect its pharmacological attributes (reduction in half-life) or structural ability to bind to the erythropoietin receptor homodimer. Non-limiting examples of suitable tissue protective cytokines for use with the present invention include the tissue protective cytokines disclosed in International Publication No. WO/02053580 and U.S. Patent Publication Nos. 2002/0086816 and 2003/0072737, which are incorporated by reference herein in their entirety.

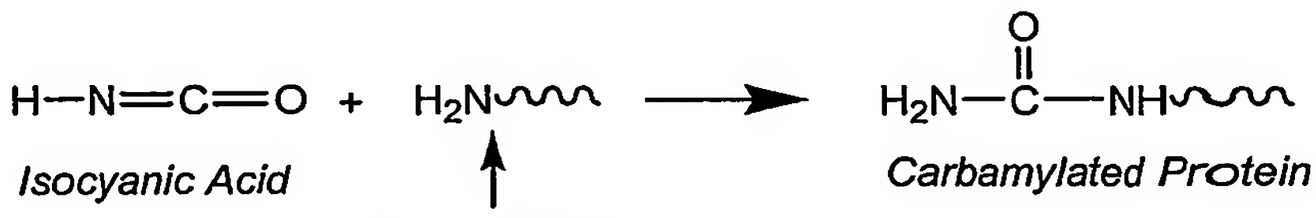
In addition, the tissue protective cytokines for use with the present invention may include EPO molecules with a modification of at least one arginine, lysine, tyrosine, tryptophan, or cysteine residue or carboxyl groups are also contemplated for use as tissue protective cytokines according to the present invention. These residues may be chemically modified by guanidination, amidination, carbamylation, trinitrophenylation, acylation (acetylation or succinylation), nitration, or mixtures thereof, as disclosed in International Publication No. WO/02053580.

Thus, the tissue protective cytokine of the present invention may be carbamylated EPO. As discussed in the background of the invention, rhu-EPO has been researched in connection with treatment of acute renal failure, which is a possible complication of septicemia. However, because rhu-EPO has erythropoietic activity, *i.e.*, the ability to maintain hematocrit levels in the body and hyperactivation of platelets, red blood cells are increased and platelets become hyperactive upon administration thereof resulting in the blood thickening and an increased risk of thrombosis. Thus, the use of rhu-EPO would likely exacerbate the widespread clotting that occurs as a result of sepsis.

Unlike rhu-EPO and selected other modified EPO molecules, carbamylated EPO does not retain erythropoietic activity and fails to bind with the classic homodimer erythropoietin receptor as is noted in PCT application no. PCT/US04/013099, filed April 26, 2004, hereby

incorporated in its entirety. Carbamylated EPO, however, does advantageously maintain the tissue protective functionality of endogenous EPO. It is believed that the retained tissue protective function of carbamylated EPO is mediated through its interaction with a tissue protective receptor complex as disclosed in PCT application no. PCT/US04/013099. Thus, carbamylated EPO may be used to treat, prevent, delay the onset, and/or reduce the effects of pro-inflammatory cytokines such as TNF within conditions including, but not limited to, sepsis, adhesions, wound healing, chronic diseases and general inflammatory conditions without posing the risk of further clotting associated with the administration of erythropoietin. In addition, because the carbamylated EPO molecules of the present invention are effective in protecting against necrosis, the carbamylated EPO molecules of the present invention are particularly beneficial in treating, preventing, delaying the onset, and/or reducing the effects of sepsis, adhesions, and general inflammatory conditions in patients susceptible to stroke, myocardial infarction, deterioration of mental faculties, and age-related conditions.

Therefore, the tissue protective cytokine of the invention may be a modified EPO with alteration of at least one or more lysine residues or the N-terminal group of the EPO molecule, which for purposes of this application, may also be referred to as "sites". The modifications may result from the reaction of the lysine residue or N-terminal amino group with an amino-group modifying agent. For example, the generic reaction scheme below is representative of one method to carbamylate proteins, such as EPO:



In another embodiment, one or more lysine residues on an EPO molecule may be carbamylated by virtue of reaction with a cyanate ion. For example, one or more lysine residues may be modified by incubation with 4-sulfophenylisothiocyanate. In yet another embodiment, one or more lysine residues on the EPO molecule are alkyl-carbamylated, aryl-carbamylated, or aryl-thiocarbamylated with an alkyl isocyanate, an aryl isocyanate, or an aryl-thioisocyanate, respectively. In still another embodiment, one or more lysine residues are akylated by a reactive alkylcarboxylic or arylcarboxylic acid derivative, *e.g.*, acetic

anhydride, succinic anhydride, or phthalic anhydride. The modified lysine residue may also be chemically reduced.

One or more lysine residues may also be carbamylated by reacting the residue(s) with trinitrobenzenesulfonic acid, or a salt thereof. In yet another embodiment, one or more lysine residues may be modified by reaction with a glyoxal or a glyoxal derivative, *e.g.*, methylglyoxal ir 3-deoxyglucosone, to form the corresponding alpha-carboxyalkyl derivatives.

Other methods of carbamylation may be used in accordance with the present invention. For example, the method disclosed in Plapp *et al.*, J. BIOL. CHEM., 246: 939-945 (1971) is a suitable way of making the carbamylated EPO according to the present invention. Another example of a method of carbamylation is discussed in Satake *et al*, 1990, *Biochim. Biophys. Acta* 1038:125-9, where six of the lysine residues in erythropoietin were carbamylated.

And, as mentioned above, any of the forms of EPO may be used according to the present invention. Thus, as an example: in one embodiment, the EPO molecule subject to carbamylation is in α form; in another embodiment, the EPO molecule subject to carbamylation is in β form; and in yet another embodiment, the EPO molecule subject to carbamylation is asialic.

The carbamylation process preferably occurs for a period of time sufficient to substantively reduce or completely eliminate erythropoietic activity. In one embodiment, the carbamylation process is performed for a sufficient time period to remove at least about 90 percent of the sites. In another embodiment, the carbamylation process is performed for a sufficient time period to remove at least about 95 percent of the sites. In still another embodiment, the carbamylation process is performed for a sufficient time period to remove 100 percent of the sites. Alternatively, this may be viewed as carbamylating erythropoietin for a period of time sufficient to carbamylate at least six lysine residues in one embodiment, at least seven lysines in another embodiment, and at least eight lysine residues in another embodiment. The time required for sufficient carbamylation to occur may vary. For instance, sufficient carbamylation may occur over a period up to about 6 to 24 hours, up to about 10 to 20 hours, or up to about 16 hours.

The tissue protective cytokines for use with the present invention may also be obtained by limited proteolysis, removal of amino groups, and/or mutational substitution of arginine, lysine, tyrosine, tryptophan, or cysteine residues by molecular biological techniques as disclosed in Satake *et al*, 1990, *Biochim. Biophys. Acta* 1038:125-9, which is incorporated

by reference herein in its entirety.. For example, suitable tissue protective cytokines include at least one or more mutated EPOs having a site mutation at C7S, R10I, V11S, L12A, E13A, R14A, R14B, R14E, R14Q, Y15A, Y15F, Y15I, K20A, K20E, E21A, C29S, C29Y, C33S, C33Y, P42N, T44I, K45A, K45D, V46A, N47A, F48A, F48I, Y49A, Y49S, W51F, W51N, Q59N, E62T, L67S, L70A, D96R, K97D, S100R, S100E, S100A, S100T, G101A, G101I, L102A, R103A, S104A, S104I, L105A, T106A, T106I, T107A, T107L, L108K, L108A, S126A, F142I, R143A, S146A, N147K, N147A, F148Y, L149A, R150A, G151A, K152A, L153A, L155A, C160S, I6A, C7A, B13A, N24K, A30N, H32T, N38K, N83K, P42A, D43A, K52A, K97A, K116A, T132A, I133A, T134A, K140A, P148A, R150B, G151A, K152W, K154A, G158A, C161A, and/or R162A. Examples of the above-referenced modifications are described in co-pending U.S. Patent Publication Nos. 2003/0104988, 20O2/0086816 and 2003/0072737, which are incorporated by reference herein in their entirety. In the mutein nomenclature used herein, the changed amino acid is depicted with the native amino acid's one letter code first, followed by its position in the EPO molecule, followed by the replacement amino acid one letter code. For example, S100E refers to a human EPO molecule in which, at amino acid 100, the serine has been changed to a glutamic acid.

In another embodiment, the tissue protective cytokine may include one or more of the above site mutations, providing that the site mutations do not include I6A, C7A, K20A, P42A, D43A, K45D, K45A, F48A, Y49A, K52A, K49A, S100B, R103A, K116A, T132A, I133A, K140A, N147K, N147A, R150A, R150E, G151A, K152A, K154A, G158A, C161A, or R162A.

In still another embodiment, the tissue protective cytokines may include combinations of site mutations, such as K45D/S100E, K97D/S100E, A30N/H32T, K45D/R150E, R103E/L108S, K140A/K52A, K140A/K52A/K45A, K97A/K152A, K97A/K152A/K45A, K97A/K152A/K45A/K52A, K97A/K152A/K45A/K52A/K140A, K97A/K152A/K45A/K52A/K140A/K154A, N24K/N38K/N83K, and N24K/Y15A. In yet another embodiment, the tissue protective cytokines do not include any of the above combinations. In another embodiment, the tissue protective cytokines may include any of the above-referenced site mutations providing that the site mutations do not include any of the following combinations of substitutions: N24K/N38K/N83K and/or A30N/H32T.

Certain modifications or combinations of modifications may affect the flexibility of the mutein's ability to bind with its receptor, such as an EPO receptor or secondary receptor. Examples of such modifications or combinations of modifications include, but are not limited to, K152W, R14A/Y15A, I6A, C7A, D43A, P42A, F48A, Y49A, T132A, I133A, T134A,

N147A, P148A, R150A, G151A, G158A, C161A, and R162A. Corresponding mutations are known to those of ordinary skill in the art to be detrimental in human growth hormone. Thus, in one embodiment, the tissue protective cytokine does not include one or more of the modifications or combinations of modifications that may affect the flexibility of the mutein's ability to bind with its receptor. Further discussion of such tissue protective cytokines is included in co-pending U.S. Patent Application No. 10/612,665, attorney docket no. 10165-022-999, filed July 1, 2003, entitled "Recombinant Tissue Protective Cytokines and Encoding Nucleic Acids Thereof for Protection, Restoration, and Enhancement of Responsive Cells, Tissues, and Organs," the entire disclosure of which is incorporated by reference herein

Moreover, suitable tissue protective cytokines for use with the present invention includes the long acting chemically modified EPO molecules disclosed in International Application No. US03/028073, filed under attorney docket no. 20528.0006 on September 9, 2003, entitled "Long Acting Erythropoietins that Maintain Tissue Protective Activity of Endogeneous Erythropoietin", which is incorporated in its entirety by reference herein. For example, suitable tissue protective cytokines for use with the present invention includes EPO that has undergone at least one chemical modification to at least one of the N-linked oligosaccharide chains or the O-linked oligosaccharide chain, wherein the chemical modification includes oxidation, sulfation, phosphorylation, PEGylation, or a combination thereof. In one embodiment, the EPO molecule subject to chemical modification is in α form. In another embodiment, the EPO molecule subject to chemical modification is in β form. In yet another embodiment, the EPO molecule subject to chemical modification is asialic. In yet another embodiment, the EPO molecule subject to chemical modification may be ARANESP (Amgen, Thousand Oaks, CA) or CERA (Hoffmann-La Roche Inc., Nutley, NJ).

A variety of host-expression vector systems may be utilized to produce the tissue protective cytokines of the present invention. For example, when the tissue protective cytokine is based on an EPO molecule, various host-expression systems may be used. Such host-expression systems represent vehicles by which EPO may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the modified erythropoietin gene product *in situ*. These include but are not limited to, bacteria, insect, plant, mammalian, including human host systems, such as, but not limited to, insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing EPO coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus,

CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing erythropoietin-related molecule coding sequences; or mammalian cell systems, including human cell systems, *e.g.*, HT1080, COS, CHO, BHK, 293, 3T3, harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells, *e.g.*, metallothionein promoter, or from mammalian viruses, *e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter.

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications and processing of protein products may be important for the function of the protein. As known to those of ordinary skill in the art, different host cells have specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells, including human host cells, include but are not limited to HT1080, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the recombinant tissue protective cytokine-related molecule gene product may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements, *e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, and the like, and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the EPO mutein-related molecule gene product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the functionality of the EPO-related molecule gene product.

Alternatively, the expression characteristic of an endogenous EPO mutein gene within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted

regulatory element is operatively linked with the endogenous erythropoietin mutein gene. For example, an endogenous EPO mutein gene that is normally "transcriptionally silent", *i.e.*, an EPO gene that is normally not expressed, or is expressed only at very low levels in a cell line, may be activated by inserting a regulatory element that is capable of promoting the expression of an expressed gene product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous EPO gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such it is operatively linked with an endogenous erythropoietin gene, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art, and also described French Patent No. 2646438, U.S. Patent Nos. 4,215,051 and 5,578,461, and International Publication Nos. WO93/09222 and WO91/06667, the entire disclosures of which are incorporated by reference herein.

Pharmaceutical Compositions

The present invention also relates to pharmaceutical compositions including the tissue protective cytokines of the present invention. Because the tissue protective cytokines of the present invention advantageously have the ability to ameliorate the effects of proinflammatory cytokines, such as TNF, as well as the ability to protect tissues from cell death, the cytokines are contemplated for the treatment of sepsis, adhesions, wounds, chronic disease and inflammatory conditions in individuals also at risk for various tissue injuries, such as stroke and myocardial infarction.

In addition, the tissue protective cytokines of the present invention are contemplated for treatment of sepsis, adhesions, wounds and general inflammatory conditions in individuals also experiencing deterioration of mental faculties, such as Alzheimer's, Parkinson's and the like.

The pharmaceutical compositions of the invention contain a therapeutically effective amount of the tissue protective cytokine of the present invention, preferably in purified form. As used herein, the term "therapeutically effective amount" means an amount of tissue protective cytokine that is nontoxic but sufficient to provide the desired effect and performance at a reasonable benefit / risk ratio attending any medical treatment.

The formulation should suit the mode of administration. In other words, the pharmaceutical compositions of the invention include an amount of the tissue protective cytokine(s) of the invention such that the targeted effects of proinflammatory cytokines, *i.e.*,

fever, wasting, lethargy, anemia, edema, ischemia, organ failure, and insulin resistance, or conditions related to proinflammatory cytokines, *i.e.*, sepsis, adhesions, wound healing, chronic disease or an inflammatory condition, is treatable provided the proper dose and strategy is employed. And, as discussed in more detail below, the pharmaceutical composition should be delivered in a non-toxic dosage amount.

In one embodiment, a chemically modified or mutated erythropoietin is included in the pharmaceutical composition of the invention. In another embodiment the chemically modified erythropoietin is a carbamylated EPO. The carbamylated EPO may be an EPO molecule with at least one or more modified lysine residues or a modified N-terminal group. In another embodiment, the mutated erythropoietin may be S100E. In addition, the present invention contemplates the use of a mixture of tissue protective cytokines produced by any of the methods of the present invention described above in the pharmaceutical compositions of the invention. For example, the pharmaceutical composition of the invention may include at least one carbamylated EPO that is a result of modifying one or more lysine residues and at least one mutated EPO that is the result of modifying an amino group within erythropoietin, such as S100E.

The pharmaceutical compositions of the invention may include a therapeutically effective amount of the tissue protective cytokine and a suitable amount of a pharmaceutically acceptable carrier so as to provide the form for proper administration to the patient. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized foreign pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered.

Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like.

The pharmaceutical compositions of the invention may also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Treatment and Administration Methods

The aforementioned tissue protective cytokines and pharmaceutical compositions including the tissue protective cytokines are intended for the therapeutic or prophylactic treatment, prevention, delay, and reduction of the effects of proinflammatory cytokines, such as TNF. These effects include fever, wasting, lethargy, anemia, edema, ischemia, organ failure and insulin resistance. For example, as demonstrated below in Example 4, the tissue protective cytokines of the present invention reduced the fever, elevation of the body's core temperature above the body's normal core temperature, associated with the release of proinflammatory cytokines. As is demonstrated in Figure 6, the administration of a tissue protective cytokine, carbamylated erythropoietin, resulted in a greater than 50% reduction in the fever experienced as a result of subjecting a rat to LPS. The tissue protective cytokines may be administered to treat, prevent, delay or reduce conditions related to proinflammatory cytokines such as sepsis and sepsis-related conditions such as adhesions.

In addition, the tissue protective cytokines of the present invention are also contemplated for the treatment and prevention of inflammatory conditions in one or more organ(s) or tissue(s). The organs include, but are not limited to, the airways and lung, the kidney and urinary tract system, and the prostrate. As used herein, the term "inflammatory condition" refers to a condition in which mechanisms such as the reaction of specific T lymphocytes or antibody with antigen causes the recruitment of inflammatory cells and endogenous mediator chemicals. In some cases, the normal function of the organ or tissue will be altered by an increase in vascular permeability and/or by contraction of visceral smooth muscle.

Thus, the tissue protective cytokines of the present invention may be used to treat and/or prevent inflammatory conditions wherein the normal function of the organ(s) or tissue(s) is altered. These conditions may include ischemia-related conditions, as well as non-ischemia-related conditions, such as allergy, rheumatic diseases, and infection including viral, fungal, and bacterial infection. Furthermore, the injury or infection may be acute or chronic. In one embodiment, the tissue protective cytokines of the invention are contemplated for use in treating and/or preventing inflammatory conditions under non-ischemia conditions, *i.e.*, conditions where there is a substantially normal blood supply to the organ(s) and/or tissue(s) in question.

Furthermore, the tissue protective cytokines of the present invention may be used to enhance the healing of wounds. This may be accomplished by reducing the time needed to heal, reducing the appearance of or completely eliminate scarring, reducing the risk of complications, or otherwise improving the quality of healing. For example, scarring from an incision may be dramatically reduced, if not completely avoided, when the tissue protective cytokines of the present invention are employed prior to, during, or after the incision occurs. In addition to surgical procedures the tissue protective cytokines of the present invention are useful in addressing wounds resulting from conditions including but not limited to trauma (blunt force and cuts), pressure (bed sores), burns, and diseases, such as diabetes or vascular insufficiencies.

Moreover, the tissue protective cytokines and pharmaceutical compositions of the present invention may be used to address the effects of proinflammatory cytokines, such as TNF. As demonstrated in Figures 7a and 7b, the tissue protective cytokines of the present invention can reduce the upregulation of proinflammatory cytokines, IL-6 and TNF respectively, in response to an injury or infective agent. The tissue protective cytokines of the present invention may be administered in therapeutic doses to treat, prevent, reduce, or eliminate effects of proinflammatory cytokines such as fever, wasting, lethargy, anemia, edema, ischemia, organ failure and insulin resistance. Given that the tissue protective cytokines interfere with the upregulation of proinflammatory cytokines, the tissue protective cytokines of the present invention may be able to restore endogenous functions interrupted by the proinflammatory cytokines without directly affecting those endogenous functions. Additionally, the tissue protective cytokines of the present invention may be administered in conjunction with other known therapeutic treatments for conditions related to proinflammatory cytokines to provide a synergistic effect. For example, a treatment for the anemia associated with cancer or other chronic diseases may involve the administration of a

typical therapeutic dose of recombinant erythropoietin to restore the patient's hematocrit and a therapeutic dose of the tissue protective cytokines of the present invention to counteract the effects of proinflammatory cytokines. This would permit the use of lower doses of recombinant erythropoietin in such chronic disease thereby greatly reducing the risk of thrombolic events.

The tissue protective cytokines of the present invention may be used for systematic or chronic administration, acute treatment, and/or intermittent administration. In one embodiment, the pharmaceutical compositions of the invention are administered chronically to protect or enhance the target cells, tissue or organ. In another embodiment, the pharmaceutical compositions of the invention may be administered acutely, *i.e.*, for a single treatment during injury. In yet another embodiment, the pharmaceutical compositions of the invention are administered in a cyclic nature.

The compositions of the invention may be administered prior to injury. As such, the tissue protective cytokines of the present invention may be administered prior to a surgical procedure to prevent sepsis, delay the onset of sepsis, and/or reduce complications from sepsis. For example, the tissue protective cytokines of the present invention may be given to a patient prior to abdominal surgery. And, as briefly mentioned above, administering the tissue protective cytokines of the present invention prior to surgery may not only have an effect with regard to sepsis, adhesions, and general inflammatory conditions, but they may also reduce the appearance of, or completely eliminate scarring from the surgery.

In addition, the compositions of the invention may be administered at the time of injury or shortly thereafter. Thus, a patient undergoing major abdominal surgery may be given the tissue protective cytokines of the present invention at the time of, or shortly thereafter, the surgical procedure in order to prevent, delay the onset of, or reduce complications stemming from sepsis, adhesions, or general inflammatory conditions. The tissue protective cytokines of the present invention may also reduce the appearance of, or completely eliminate scarring from the surgery if administered during or after injury.

For example, the tissue protective cytokines of the present invention may be used for irrigation purposes, *e.g.*, while cleaning the wound, a saline solution including the tissue protective cytokine of the present invention may be administered to treat, prevent, delay the onset of, or reduce complications stemming from sepsis, adhesions, or general inflammatory conditions. Furthermore, the tissue protective cytokines of the present invention may be given to a pregnant woman following a cesarean section in order to prevent, delay the onset of, and/or reduce complications from sepsis, adhesions, and/or general inflammatory

conditions. As another example, the tissue protective cytokines of the present invention may be given to a patient during chemotherapy to stave off sepsis, adhesions, or general inflammatory conditions.

In one embodiment, the tissue protective cytokines of the present invention are administered intravenously at the time of injury and subcutaneously for a predetermined period of time thereafter in order to prevent, delay the onset of, or reduce complications stemming from sepsis, adhesions, or general inflammatory conditions. For example, the compositions of the invention may be administered in an amount of about 10 μg/kg intravenously at the time of injury followed by 10 μg/kg subcutaneously for an allotted time.

In cases of a positive sepsis diagnosis, the compositions of the invention may be administered daily to treat sepsis, stabilize the patient, and prevent the sepsis condition from progressing to a more serious stage, *e.g.*, severe sepsis or septic shock. In addition, the tissue protective cytokines of the present invention may be administered with known antibiotics, anti-fungals, anti-virals, and the like, including those listed within International Publication No. WO 2004/004656, hereby incorporated by reference in its entirety.

The administration of the composition may be parenteral, *i.e.*, by a method other than the via the digestive tract. For example, parenteral administration may include intravenous injection, intraperitoneal injection, intra-arterial, intramuscular, intradermal, or subcutaneous administration. The composition may also be administered via inhalation or transmucosally, *e.g.*, orally, nasally, rectally, intravaginally, sublingually, submucosally, and transdermally. In addition, the tissue protective cytokines of the present invention may be administered locally to the area in need of treatment, such as by the use of a perfusate; topical application, *e.g.*, in conjunction with a wound dressing after surgery; by injection; by means of a catheter; by means of a suppository; or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers. Combinations of the administration methods discussed above are contemplated by the present invention.

In one embodiment, the administration of the pharmaceutical composition of the invention is parenteral. Such administration may be performed in a dose amount of about 0.01 pg to about 5 mg, preferably about 1 pg to about 5 mg. In one embodiment, the dose amount is about 500 pg to about 5 mg. In another embodiment, the dose amount is about 1 ng to about 5 mg. In yet another embodiment, the dose amount is about 500 ng to about 5 mg. In still another embodiment, the dose amount is about 1 μg to about 5 mg. For example, the dose amount may be about 500 μg to about 5 mg. In another embodiment, the dose

amount may be about 1 mg to about 5 mg. Such compositions may include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially isotonic with the blood of an intended recipient. In this aspect of the invention, the pharmaceutical compositions may also include water, alcohols, polyols, glycerine, vegetable oils, and mixtures thereof.

Pharmaceutical compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a lyophilized (freeze-dried) condition requiring only the addition of a sterile liquid carrier, *e.g.*, sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. In one embodiment, an autoinjector comprising an injectable solution of a long acting EPO of the invention may be provided for emergency use by ambulances, emergency rooms, and battlefield situations.

Intravenous Administration

In one embodiment, the pharmaceutical composition of the invention is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. For example, the pharmaceutical composition may be in the form of a solution in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical composition may also include a solubilizing agent and/or a local anesthetic such as lidocaine to ease pain at the site of the injection. The ingredients may be supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent. When the pharmaceutical compositions of the invention are to be administered by infusion, an infusion bottle with sterile pharmaceutical grade water or saline may be used for dispensing the composition. And, when the pharmaceutical composition are to be administered by injection, an ampule of sterile saline may be provided to mix the ingredients may be mixed prior to administration.

Oral Administration

One of ordinary skill in the art will recognize the pharmaceutical compositions of the present invention may be adapted for oral administration as capsules or tablets; powders or granules; solutions, syrups or suspensions (in aqueous or non-aqueous liquids); edible foams or whips; emulsions; or combinations thereof. The oral formulation may include about 10

percent to about 95 percent by weight active ingredient. In one embodiment, the active ingredient is included in the oral formulation in an amount of about 20 percent to about 80 percent by weight. In still another embodiment, the oral formulation includes about 25 percent to about 75 percent by weight of the active ingredient.

Tablets or hard gelatine capsules may include lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatine capsules may include vegetable oils, waxes, fats, semi-solid, liquid polyols, or mixtures thereof. Solutions and syrups may include water, polyols, sugars, or mixtures thereof.

Moreover, an active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract. For example, the active agent may admixed or coated with glyceryl monostearate, glyceryl distearate, or a combination thereof. Thus, the sustained release of an active agent may be achieved over many hours and, if necessary, the active agent can be protected from being degraded within the stomach. Pharmaceutical compositions for oral administration may also be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

Transdermal Administration

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. In addition, pharmaceutical compositions adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols, oils, eye drops, lozenges, pastilles, and mouthwashes and combinations thereof. When the topical administration is intended for the skin, mouth, eye, or other external tissues, a topical ointment or cream is preferably used. And, when formulated in an ointment, the active ingredient, *i.e.*, the long acting EPO, may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. When the topical administration is in the form of eye drops, the pharmaceutical compositions of the invention preferably include the active ingredient, which is dissolved or suspended in a suitable carrier, *e.g.*, in an aqueous solvent.

Nasal and Pulmonary Administration

Pharmaceutical compositions adapted for nasal and pulmonary administration may include solid carriers such as powders (preferably having a particle size of about 20 microns to about 500 microns). Powders may be administered by rapid inhalation through the nose from a container of powder held close to the nose. In an alternate embodiment, pharmaceutical compositions intended for nasal administration according to the present invention may include liquid carriers, *e.g.*, nasal sprays or nasal drops. Preferably, the pharmaceutical compositions of the invention are administered into the nasal cavity directly.

Direct lung inhalation may be accomplished by deep inhalation through a mouthpiece into the oropharynx and other specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient. Pharmaceutical compositions intended for lung inhalation may include aqueous or oil solutions of the active ingredient. Preferably, the pharmaceutical compositions of the invention are administered via deep inhalation directly into the oropharynx.

Rectal and Vaginal Administration

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. In one embodiment, the suppositories of the invention includes about 0.5 percent to 10 percent by weight of active ingredient. In another embodiment, the suppository includes about 1 percent to about 8 percent by weight active ingredient. In still another embodiment, the active ingredient is present in the suppository in an amount of about 2 percent to about 6 percent by weight. In this aspect of the invention, the pharmaceutical compositions of the invention may include traditional binders and carrier, such as triglycerides.

Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Perfusion Administration

The pharmaceutical compositions of the invention may also be administered by use of a perfusate, *i.e.*, pumping a liquid into an organ or tissue (especially by way of blood vessels). In such embodiments, the pharmaceutical composition preferably has about 0.01 pM to about 30 pM, preferably about 15 pM to about 30 nM, of the tissue protective cytokine of the present invention. In one embodiment, the perfusion solution is the University of Wisconsin (UW) solution (with a pH of about 7.4 to about 7.5 and an osmolality of about 320 mOSm/l),

which contains about 1 U(10ng)/ml to about 25 U(250ng)/ml of an EPO compound of the present invention; 5 percent hydroxyethyl starch (preferably having a molecular weight from about 200,000 to about 300,000 and substantially free of ethylene glycol, ethylene chlorohydrin, sodium chloride, and acetone), 25 mM KH_2PO_4, 3 mM glutathione; 5 mM adenosine; 10 mM glucose; 10 mM HEPES buffer; 5 mM magnesium gluconate; 1.5mM $CaCl_2$; 105 mM sodium gluconate; 200,000 units penicillin; 40 units insulin; 16 mg dexamethasone; and 12 mg phenol red. The UW solution is discussed in detail in U.S. Patent No. 4,798,824, which is incorporated in its entirety by reference herein.

Local Administration

It may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment. Such administration may be achieved by local infusion during surgery; topical application, *e.g.*, in conjunction with a wound dressing after surgery; by injection; by means of a catheter; by means of a suppository; or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers.

Controlled-Release Systems

In addition, as briefly discussed above with respect to transdermal administration, the tissue protective cytokines of the present invention may be delivered in a controlled-release system. For example, the tissue protective cytokine may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. Such controlled release systems may be placed in proximity of the therapeutic target, *i.e.*, the target cells, tissue or organ, thus requiring only a fraction of the systemic dose.

Dosing

Selection of the preferred effective and non-toxic dose for the administration methods above will be determined by a skilled artisan based upon factors known to one of ordinary skill in the art. Examples of these factors include the particular form of tissue protective cytokine; the pharmacokinetic parameters of the tissue protective cytokine, such as bioavailability, metabolism, half-life, etc. (provided to the skilled artisan); the condition to be treated; the benefit to be achieved in a normal individual; the body mass of the patient; the method of administration; the frequency of administration, *i.e.*, chronic, acute, intermittent;

concomitant medications; and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and the circumstances of the particular patient.

Treatment Kits

The invention also provides a pharmaceutical pack or kit that include one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. In one embodiment, the effective amount of the tissue protective cytokine and a pharmaceutically acceptable carrier may be packaged in a single dose vial or other container.

When the pharmaceutical composition of the invention is adapted for parenteral administration, for example, the composition may be stored in a lyophilized condition. Thus, the kit may include the lyophilized composition, a sterile liquid carrier, and a syringe for injections.

In one embodiment, the kit includes an ampule containing enough lyophilized material for several treatments such that the administrator would weigh out a specific amount of material and add a specific amount of carrier for each treatment session. In another embodiment the kit may contain a plurality of ampules each containing specific amounts of the lyophilized material and a plurality of containers each containing specific amounts of carrier, such that the administrator need only mix the contents of one ampule and one carrier container for each treatment session without measuring or weighing. In yet another embodiment, the kit contains an autoinjector including an injectable solution of the tissue protective cytokine(s) of the invention. In still another embodiment, the kit contains at least one ampule with the lyophilized composition, at least one container of carrier solution, at least one container with a local anesthetic, and at least one syringe (or the like). The ampules and containers are preferably hermetically-sealed.

When the pharmaceutical compositions of the invention are to be administered by infusion, the kit preferably includes at least one ampule with the pharmaceutical composition and at least one infusion bottle with sterile pharmaceutical grade water or saline.

A kit according to the present invention may also include at least one mouthpiece or specially adapted devices for direct lung inhalation such as pressurized aerosols, nebulizers, or insufflators. In this aspect of the invention, the kit may include the device for direct lung inhalation, which contains the pharmaceutical composition, or the device and at least one

ampule of aqueous or oil solutions of the tissue protective cytokine(s) of the present invention.

When the tissue protective cytokine(s) of the invention is adapted for oral, transdermal, rectal, vaginal, or nasal, the kit preferably includes at least one ampule containing the active ingredient and at least one administration aid. Examples of administration aids include, but are not limited to, measuring spoons (for oral administration), sterile cleaning pads (for transdermal adminstration, and nasal aspirators (for nasal administration). Such kits may include a single dose of the tissue protective cytokine (acute treatment) or a plurality of doses (prolonged treatment).

In addition, the kit may be outfitted with one or more types of solutions. For example, the tissue protective cytokines of the invention may be made in an albumin solution and a polysorbate solution. If the kit includes the polysorbate solution, the words "Albumin free" preferably appear on the container labels as wells as the kit main panels.

Moreover, the kit may also include a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Assays to Determine Sepsis / Inflammation Treatability

The present invention also contemplates assays to determine whether a tissue protective cytokine is able to effectively treat, prevent, delay the onset of, or reduce complications of sepsis, adhesions, and inflammation resulting from infection. Any assay that includes laboratory controlled sepsis induction, adhesion induction, or inflammatory response induction is contemplated for the present invention.

For example, a suitable assay according to the invention may include a blind study where the cecum of Sprague Dawley rats are exposed and ligated just distally to the ileocecal valve to avoid intestinal obstruction. The cecum is then be punctured and squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity. The release of feces into the organ induces infection which, in turn, induces sepsis, adhesions, inflammation, or a combination thereof. The abdomen is then sutured. The rats are preferably separated into groups with at least one group receiving saline and another group receiving the tissue protective cytokine to be tested. At the time of ligation, the various groups of animals are given a predetermined amount of saline or the tissue protective cytokine, preferably intraveneously. Subcutaneous administration of the selected treatment, *i.e.*, saline or the

tissue protective cytokine, may be undertaken for a predetermined time following the ligation procedure. In addition, the study may include a group of rats that have been opened, but not subjected to infection.

The animals may then be monitored for adhesions and illness scores. Table 1 provides a method of scoring an animal based on the formation of adhesions.

TABLE 1. CUMULATIVE ADHESION SCORING SCALE

Points	0	No adhesions
	+ 1	One adhesive band from the omentum to the target organ
	+ 1	One adhesive band from the omentum to the scar
	+ 1	One adhesive band from the omentum to the another place
	+ 1	One adhesive band from adnexa / epididymal fat bodies to the target organ
	+ 1	One adhesive band from adnexa / epididymal fat bodies to scar
	+ 1	One adhesive band from adnexa / epididymal fat bodies to another place
	+ 1	Any adhesive band other than described above (e.g., liver to scar)
	+ 1	Target organ adherent to abdominal wall
	+ 1	Target organ adherent to abdominal scar
	+ 1	Target organ adherent to bowel
	+ 1	Target organ adherent to liver or spleen
	+ 1	Any other organ adherent
Total Score		

One point is given for each adhesion and a cumulative adhesion score is calculated. In one embodiment, the cumulative adhesion score is preferably 8 or less. In another embodiment, the cumulative adhesion score is about 5 or less. In still another embodiment, the cumulative adhesion score is about 3 or less.

An illness score may also be calculated for each animal based on a variety of factors. Factors used for this score include, but are not limited to, behavioral factors such as walking posture, rope hanging ability, investigatory behavior regarding surroundings, climbing foam pad up a wall, body responses such as erectness of hair, and oxygenation, and the number of adhesions formed. For example, when a rat is ill, the animal will hunch while walking and will not investigate his/her surroundings. In addition, the pulse rate of an ill rate is inaccurate, while a healthy rat typically has a pulse rate of about 300 beats/minute. Table 2 provides a method of scoring each animal with regard to illness in order to assess the effectiveness of the tissue protective cytokine being used for treatment

TABLE 2. CUMULATIVE ILLNESS SCORE	
Behavioral Tests	
+ 1	piloerection (hairs stand to erect)
+ 1	immobility
+ 1	Loss of Beam Balance
+ 1	unable to hold or climb
+ 1	Not using claws
+ 1	Becoming hunchbacked
+ 1	Abnormal walking
+ 1	No exploration of surroundings
+ 1	Not grasping a string within 30 seconds
+ 1	reduced reflexes
+ 1	Lack of appetite (food and drink)
+ 1	Loss of body weight
+ 1	moribund
+ 1	Abnormal Heart Rate (< or > 50% normal)
+ 1	Spontaneous Hemorrhage
+ 1	Decreased Oxygen Saturation
Total Score	

On a continuum, a cumulative illness score of 14 or above signifies the death of the animal, while a much lower score indicates that the animal is relatively healthy. In one embodiment, a sepsis-induced animal has an illness score of about 5 or less after 8 days of treatment with at least one tissue protective cytokine of the invention. In another embodiment, an animal has an illness score of about 4 or less after treatment. In yet another embodiment, an animal has an illness score of about 2 or less after treatment. In still another embodiment, an animal has an illness score of about 1 or less after treatment.

EXAMPLES

The following non-limiting examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention, the scope of which is defined by the appended claims. Parts are by weight unless otherwise indicated.

Example 1: Blind Study Using Rat Abdominal Sepsis Model

The cecum of Sprague Dawley rats was exposed, ligated just distally to the ileocecal valve to avoid intestinal obstruction, punctured twice with a 18-gauge needle, squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity (feces

introduced in peritoneum, which induced infection). The abdomen was closed with 3-0 silk sutures.

The animals were allocated to two groups:

Group 1: Sepsis induced, treated with saline (n = 8). At time of ligation, animals in Group 1 were given 100 μl saline intraveneously. Daily subcutaneous saline administration (100 μl) followed for 8 days or until death.

Group 2: Sepsis induced, treated with carbamylated EPO (n = 8). At time of ligation, animals were given 10 μg/kg carbamylated EPO (prepared so that erythropoietic activity is effectively eliminated) intraveneously in 100 μl of saline. Daily subcutaneous treatment followed for 8 days (or until death) at a dosage amount of 10 μg/kg in 100 μl saline.

Morbidity and Mortality

In Group 1, less than about 50 percent of the animals survived after 8 days. In Group 2, however, the survival rate was greater than about 50 percent, as illustrated graphically in FIG. 1. In particular, one day following the treatment there was about a 60 percent survival rate for animals in Group 1 compared to about a 80 percent survival rate for animals in Group 2. After 3 days, however, the survival rate of Group 1 dropped significantly to about 25 percent survival rate, whereas the survival rate of Group 2 animals was greater than about 60 percent. Thus, the animals receiving the carbamylated EPO of the present invention had a much higher survival rate than animals receiving saline.

Cumulative Adhesion Score

Specimens were taken from the peritoneal fluid and abscesses for aerobic and anaerobic culture. For aerobic culture, samples were incubated on blood on EMB agar for 24 hours at 37°C. For anaerobic culture, samples were layered on anaerobic blood agar and incubated in a Gas-Pak jar for 24 hours at 37°C. Growing colonies were identified with standard bacteriologic techniques.

Dead animals were autopsied within 4 hours and the causes of death were recorded. Using Table 1 described earlier in the application, a cumulative adhesion score was calculated for each animal 24 hours post-injury and then averaged for the group (shown graphically in FIG. 2). In particular, the average total score of Group 1 was about 10, whereas the average total score of Group 2 was about 6. In sum, the animals receiving the carbamylated EPO of the present invention had less adhesions than animals receiving saline.

Illness Score

An illness score was calculated as described earlier in the application in Table 2 and the results are illustrated graphically in FIG. 3. In particular, one day following the treatment, the average illness score of Group 1 animals was about 9 compared to an average illness score of Group 2 animals of about 3. After 5 days, the Group 1 animals had an average illness score of about 12, whereas the Group 2 animals had an average illness score of about 5 or less.

Scarring

The rats were also visually examined for scarring from the incisions. Group 2 rats had less scarring than Group 1 rats.

Example 2: Blind Study Using Rat Abdominal Sepsis Model

The cecum of Sprague Dawley rats was exposed, ligated just distally to the ileocecal valve to avoid intestinal obstruction, punctured twice with a 18-gauge needle, squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity (feces introduced in peritoneum, which induced infection). The abdomen was closed with 3-0 silk sutures.

The animals were allocated to three groups:

Group 1: Opened as described above, but no sepsis induced (n = 6).

Group 2: Sepsis induced, treated with saline (n = 8). At time of ligation, animals in Group 2 were given 100μl saline intraveneously. Daily subcutaneous saline administration (100 μl) followed for 8 days (or until death).

Group 3: Sepsis induced, treated with carbamylated EPO (n = 8). At time of ligation, animals were given 10 μg/kg carbamylated EPO in 100 μl saline intraveneously. Daily subcutaneous treatment followed for 8 days (or until death) at a dosage amount of 10 μg/kg in 100 μl saline.

Cumulative Adhesion Score

Specimens were taken from the peritoneal fluid and abscesses for aerobic and anaerobic culture. For aerobic culture, samples were incubated on blood on EMB agar for 24 hours at 37°C. For anaerobic culture, samples were layered on anaerobic blood agar and incubated in a Gas-Pak jar for 24 hours at 37°C. Growing colonies were identified with standard bacteriologic techniques.

Dead animals were autopsied within 4 hours and the causes of death were recorded. Using Table 1 described earlier in the application, a cumulative adhesion score was calculated for each animal 24 hours post-injury and then averaged for the group (shown graphically in FIG. 4). In particular, the average total score of Groups 1, 2, and 3 were less than about 2, about 10 and about 6, respectively. Thus, the animals receiving the carbamylated EPO of the present invention had less adhesions than animals receiving saline.

Tumor Necrosis Factor Study

The level of tumor necrosis factor (TNF) present in the blood of animals after a period of time was examined using an ELISA from R&D Systems (#RTA00) capable of to detecting rat TNF-alpha for each group in an effort to determine a mechanism behind carbamylated EPO's ability to decrease adhesions. As shown in FIG. 5, the difference in the amount of TNF after 24 hours is not significantly different between the three groups. After three hours (peak inflammation), the amount of TNF in the system decreased for all three groups. These results suggest that the accepted mechanism behind adhesions, *i.e.*, an inflammatory response, may not be the accurate mechanism. In fact, the TNF study suggests that the mechanism behind adhesion may be due to cell death and, because the carbamylated EPO of the present invention has a tissue protective function, the adhesions may decrease upon administration because of decreased cell necrosis.

Scarring

Upon visual examination, the rats in Group 3 had substantially less scarring than Groups 1 and 2.

Example 3: Blind Study Using Abdominal Sepsis Model

The cecum of Sprague Dawley rats was exposed, ligated just distally to the ileocecal valve to avoid intestinal obstruction, punctured twice with a 18-gauge needle, squeezed gently to force out a small amount of feces, and then returned to the abdominal cavity (feces introduced in peritoneum, which induced infection). The abdomen was closed with 3-0 silk sutures.

The animals were allocated to four groups:

Group 1: Opened as described above, but no sepsis induced (n = 6).

Group 2: Sepsis induced, treated with saline (n = 8). At time of ligation, animals in Group 2 were given 100 μl saline intraveneously. Daily subcutaneous saline administration (100 μl) followed for 8 days (or until death).

Group 3: Sepsis induced, treated with rhu-EPO (n=8). At time of ligation, animals were given 10 μg/kg rhu-EPO intraveneously in 100 μl saline. Daily subcutaneous treatment followed for 8 days (or until death) at a dosage amount of 10 μg/kg in 100 μl of saline.

Group 4: Sepsis induced, treated with carbamylated EPO (n = 8). At time of ligation, animals were given 10 μg/kg carbamylated EPO intraveneously in 100 μl saline. Daily subcutaneous treatment followed for 8 days (or until death) at a dosage amount of 10 μg/kg in 100 μl saline

Morbidity and Mortality

One day following ligation, all of the animals in Group 1 (sham) survived, whereas none of the animals in Group 2 survived. Only 2 of the animals in Group 3 (rhu-EPO) survived compared to 5 animals in Group 4. Thus, the animals receiving the carbamylated EPO of the present invention had a much higher survival rate than animals receiving saline or rhu-EPO.

Scarring

Upon visual examination, the rats in Group 4 had substantially less scarring than Groups 1-3.

Example 4: Lipopolysaccharide induced response in Rats

The purpose of this example was to determine the effectiveness of carbamylated erythropoietin on sepsis-like symptoms induced by lipopolysaccharide (LPS). LPS is an endotoxin present on the surface of bacteria which induces sepsis-like response (core temperature increase and cytokine induction) in animals. Male Sprague/Dawley rats (300-350g) were administered 240 ug/kg, i.p. The animals were then treated with saline (n=6) or carbamylated erythropoietin (n=6) at 10 ug/kg, i.v. The concentration dependent effects of LPS on core body temperature were then determined. Alternatively, the direct intraventricular application (Seeley et al, (1996) *Horm Metab Res*. **28**:664-8.) of carbamylated erythropoietin (5 ug/kg in 2 ul) was administered to determine if the route of application alters the core temperature differentially. Core temperature will be monitored

during the first 24 hrs. In some cases, blood was removed for subsequent cytokine (e.g., TNF, IL-6) analysis. Seeley et al, (1996) *Horm Metab Res*. **28**:664-8.

Core Temperature.

As part of the sepsis-like conditions induced by LPS, the animals administered LPS experience a biphasic fever. The first phase of the fever is characterized by a precise increase in temperature accompanied by an increase in blood pressure and wakefulness of the afflicted individual. Whereas the second phase of the fever is less precise, and is accompanied by either normotension or hypotension as well as lethargy and sleepiness. It is theorized that the phases of the fever represent a transition in the strategy that the body utilizes to combat the sickness. (Romanovsky *et al.*, *Am. J. Physiol,* 271: R244-R253, 1996) In the present example, the administration of carbamylated erythropoietin to LPS treated animals resulted in a reduction in both phases of the fever as demonstrated by Figure 6.

Serum levels of TNF and Il-6.

Carbamylated erythropoietin's ability to mediate the fever response to LPS was further correlated by its ability to suppress the presence of pyrogenic cytokines such as TNF and IL-6, as demonstrated in Figure 7(a) and Figure 7(b). ELISA was used to determine the presence of TNF and IL-6 within the serum sampled from the rats. Both Figures 7(a) and 7(b) demonstrate that the treatment with carbamylated erythropoietin significantly reduced the presence of pro-inflammatory cytokines, IL-6 and TNF.

Peripheral v. Central Administration of Carbamylated Erythropoietin.

In order to rule out the direct effect of carbamylated erythropoietin upon the hypothalamus, the carbamylated erythropoietin was administered intraventricularly in the manner noted above. As Figure 8(a) and Figure 8(b) demonstrate, there was no correlation in the reduction of core temperature between the peripherally and centrally administered carbamylated erythropoietin. In light of these results as well as carbamylated erythropoietin's effects on the pyrogenic cytokines, it appears that carbamylated erythropoietin effects the core temperature through the suppression of the pyrogenic cytokines. This suggests that tissue protective cytokines, such as carbamylated erythropoietin, are useful in mediating, ameliorating or preventing the effects of these cytokines in chronic illnesses (wasting, lethargy, anemia, etc.).

Example 5: Effect of Erythropoietin analogues on Ischemic Skin Flap Injury in Rats

An ischemic wound flap model was performed to determine the effect of carbamylated erythropoietin on ischemic skin flap wound recovery. Male Sprague/Dawley rats (300-350g) were anesthetized using isoflurane. A skin flap 9 cm long and 3 cm wide was then cut in the back of the rat. The flap included skin, subcutaneous layer and panniculus carnosus. Following incision, the flap was raised and then immediately re-sutured in its bed, as described (Buemi, M., et al., (2002) *Acta Derm. Venereol.* **82**:411-417; Sarau, A., et al., (2003) *Laryngoscope.* **113**:85-89). Animals were dosed with an erythropoietin analogue, carbamylated EPO, (0.3 μg/kg, s.c.) immediately following surgery, day 1, day 2 and then bi-weekly during analysis. Animals were weighed and the wound photographed weekly. Buemi, M., et al., (2002) *Acta Derm. Venereol.* **82**:411-417. The area of the wound healed was then quantified based on photographs of the animals taken 34 days following the proceedure. As is demonstrated in Figure 9, the rats that received carbamylated erythropoietin had a greater percentage of the wound healed than those treated with saline for the same time period.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. In particular, one of ordinary skill in the art will recognize that althought the above examples were performed using carbamylated EPO, similar results would be expected of any of the tissue protective cytokines of the present invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. All patents and patent applications cited in the foregoing text are expressly incorporate herein by reference in their entirety.